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Patentanmeldung Nr. Patent application No. Demande de brevet n°

97204110.7

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Preparation of cells for production of biologicals

The present invention is concerned with a method for the preparation of cells for use in the production of biologicals.

For the production of biologicals on e.g. cell lines, the preparation of large amounts of cells using an scaling up procedure in bioreactors will be necessary.

The US patent No. 5,017,490 discloses such a scaling up procedure which provides in particular the advantage of a low risk of transfer contamination. This method is, however, not suited for anchorage dependent cells (hence, not for cells which only grow if fixed to a substrate) or cells embedded in a substrate (e.g. in porous carriers).

The US patent No. 4,644,912 discloses a method for the preparation of anchorage-dependent cells for the production of biologicals (i.e. viruses) starting with a cell working seed, and with subsequent passages effected in increasing consecutive volumes of 1 litre, 5 litre, 25 litre, 150 litre bioreactors, and finally either in a 1000 litre bioreactor or in a multiplicity of 150 litre bioreactors. In between any of these passage steps the cells were released from their carriers with a dilute protease solution. In the final passage the inoculation by the virus was effected.

Assuming average cell cycle times of about 20-24 hours the passage intervals may be about every 3-5 day. Therefore, in order to expand the cells to sufficient large cultures from a MWCS[1] the total scaling up procedure may take several weeks, depending on the final bioreactor volume.

In the above methods for preparation of cells each of the ultimate production batches has to be prepared from the MWCS. For the production of vast amounts of biologicals it will be necessary to utilise several parallel culturing lines up to the largest vessel volumes. Such preparation procedure, hence, is very time consuming and necessitates the operation of a very considerable number of bioreactors for the preparation of the cells as well as for the production of the biologicals.

[1] MWCS = manufacturer's working cell bank

It is an object of the present invention to provide a much faster through-put in preparation of cells for the production of biologicals.

Accordingly, the present invention relates to a method for the preparation of cells for use in the production of biologicals, by culturing cells from a working seed stock by one or more passages up to a defined maximum number of passages into a preproduction batch in a mother bioreactor wherein from the mother bioreactor in repeating steps:
(1) part of the content of the mother bioreactor is transferred to one or more production bioreactors, and
(2) the remaining content of the mother bioreactor is used as a seed for subsequent preparation of a preproduction batch.

The basic method of working is to use a mother bioreactor from which the production bioreactor(s) is(are) fed with cells. When the cells are anchorage dependent, after each passage step cells need to be detached from their substrates.
A trypsinisation procedure on large bioreactors has been developed for this purpose.
The production cells are defined up to a defined and characterised passage number for a so-called ECB[2]. The method described allows high through-put production since the up scaling route can be omitted and much less bioreactors are needed since parallel production lines are not needed anymore.

Various embodiments of the present invention are depicted in Figure 1.

In a preferred embodiment cells are expanded from one ampoule of a MWCS up to the level of the "mother bioreactor" through one or more passage steps. The size of such bioreactor can range from several litres working volume to several hundreds of litres. Next, a part e.g. 10-20 % of the cells thus expanded (e.g. passage X) are used to repopulate the "mother bioreactor" (being passage number X+1), whereas the bulk of the cells is transferred (passage X or X+1) to a larger bioreactor size in order to start production directly or to first populate it, subsequently start production.

In classical serial production lines the number of doubling of the cells derived from the MWCS at the moment of harvest is known up front within certain limits. A maximum allowable generation number is set to the production system at the onset.

[2] ECB = Extended Cell Bank

In the method according to the present invention the maximum number of cell passages is defined by ECB. Production passage number (the number of cell passages used prior to production of the biological product), hence, is irrelevant within the limits set by ECB. As a consequence, such maximum number of passages is to be obeyed in view of regulatory restrictions. As a result the particular batch of produces biologicals is the end product of one direct scaling up route.

In order to verify whether the specifications of the cells at the stage of ECB in production are similar to the MCB[3] one need to perform specific validation for this purpose with respect to growth characteristics, freedom of adventitious and endogenous agents at the different stages, karyology isoenzyme analysis and so on. Once such ECB is fully characterised one may allow to produce the product with cells at any passage number between MCB and ECB, since it may be assumed that cells have not changed in between in their specs. As a result tests on the MWCS therefor can be limited to sterility testing. This is a particular advantage of the method according to the present invention

With the maximum passage number set one may use cells at any stage in between. From this in order to further minimise the time needed to expand the cells from the MWCS to production bioreactor it would be an advantage to enable bulk start-up of cells. This can be done for example in one of the following ways:

- Cells may be parked at a certain passage number during longer intervals at ambient temperature (17-32 °C) and be revitalised to log expansion growth by raising the temperature and changing the culture medium, or
- Cells may be frozen (Temp < -80°C) in bulk and be thawed prior to transfer them to a pre-set volume bioreactor, thereby reducing the needed up scaling route significantly.

The method according to the present invention can be carried out with animal cell cultures and more in particular with anchorage dependent cells. Suitable types of cells are e.g. hamster cells (CHO, BHK-1), monkey cells (Vero), bovine cells (MDBK), canine cells (MDCK), human cells (CaCo, A431) or chicken cells (CEF).

[3] MCB = Master Cell Bank

As a bioreactor according to the present inventions can be used a single unit of a plurality of units of e.g. stirred fermenters, fixed bed fermenters, fluidised bed fermenters, air lift fermenters, or a hollow fibre reactors.

Cells of the above times can and some even should be cultured when fixed to a solid support, like microcarriers or macrocarriers in suspension, e.g. in a fixed bed, a fluidised bed or in suspension, or like hollow fibres. Cells can also be embedded into a carrier (e.g. porous carrier)

In the course of the method according to the present invention, in particular when using a solid support, cells are to be released from this solid support. This can be effected by any method useful for detaching of cells from a solid support. Advantageously, to this end use can be made of a proteolitic enzyme solution. Optionally, this enzymatic release step can be preceded by one or more pre-conditioning steps, e.g. by treatment with PBS and/or EDTA, in order to enhance the proteolitic efficiency, and/or in order to reduce the amount of proteolitic enzyme required.

EXAMPLE 1

Cell detachment and separation from carriers prior to transfer to next bioreactor

Anchorage dependent cells of a MDCK[4] cell line were cultured at 37 °C on Cytodex-3 micro carriers (Pharmacia, Upsala, Sweden) (5 g of carriers/l) in a stirred bioreactor of 4 litre ("mother bioreactor"). The growth medium was EfiSerf (Life Technologies, Paisly, Scotland). Growth was continued till a maximum of $5x10^6$ cells/ml of culture.

The cells were detached from the carriers by trypsinisation in a Trypsin-EDTA solution (Life Technologies, Paisly, Scotland).

After settling of the carriers 80% of the detached cells were transferred to 3 other bioreactors of similar size. The latter "production" bioreactors all have carriers (cell substrate) added to them up front. Cells were allowed to repopulate the carriers and subsequently used for production in these production bioreactors.

The remainder of the cells in the "mother bioreactor" were allowed to repopulate the remaining Cytodex-3 carriers and were cultured to the desired cell density.

[4] MDCK = Madin Darby Canine Kidney (cell line)

EXAMPLE 2

Cell detachment without separation from carriers prior to transfer to next bioreactor

The culturing of cells was carried out as described in Example 1, however after trypsinisation 80% of the detached cells including the carriers are transferred to the 3 production bioreactors. Additionally, suitable carriers were added to all bioreactors.

EXAMPLE 3

Cell detachment without separation from carriers after transfer to next bioreactor

The culturing of cells was carried out as described in Example 1, however, 80 % of still adhered cells were transferred to a bioreactor of similar size which next was used directly for product generation.
The remaining cells on micro carriers in the mother fermenter were next detached by trypsinisation, where after new carriers were added and cells were allowed to repopulate the substrates.

EXAMPLE 4

Start-up from frozen bulk cells

In this experiment part of the culture was used to rebatch the mother fermenter and some daughter fermenters and part of the culture was used to freeze cells in bulk.
Frozen bulk cells (total $14.4X10^8$ cells) were inoculated in a start culture in a 3 litre mother fermenter containing 5 g Cytodex per litre and EfiSerf medium, and thereafter incubated at 37 °C. Residual cryo-preservatives were removed by a medium change on day 1.
At day 2 trypsinisation was carried out, 50% of the cells were bulk frozen and the remaining cells were inoculated to microcarriers in a subsequent fermenter.

On day 4 the content of the mother fermenter was trypsin-detached and rebatched onto two other fermenters next to the mother fermenter.

At day 5 the plating efficiency turned out to be about 85%.

day	3 litre fermenter cells x 100.000/ml	3 litre fermenter cells x 100.000/ml	3 litre fermenter cells x 100.000/ml
0	ND		
1	6.6		
2	14		
3	15.5		
4	30		
5	5.5	10	10

EXAMPLE 5

Transfer from small scale mother fermenter to large scale production fermenter

Cells were scaled up to a large scale in 65 litre and 550 litre fermenters (50 litre and 250 litre working volume, respectively) using a microcarrier density of 5 g Cytodex per litre.
As can be seen from the table 90 % of the viable cells is transferred to the large scale fermenter from a 50 litre fermenter culture with 800.000 cells/ml.
The procedure was as follows:
On day 0, the carriers were allowed to settle in the 50 litre culture, where after the supernatant (culture medium) was removed and replaced by PBS. The content of the fermenter was agitated for 5-15 minutes. The supernatant was removed after resettling of the carriers. This step can be repeated if needed.
Next this step was repeated with PBS/EDTA (0.4 gram EDTA/litre PBS). Again the culture was agitated during 5-15 minutes, carriers were allowed to settle, the supernatant was removed, and the PBS/EDTA step was repeated until cells had become rounded and were ready to be trypsin-detached.
Then trypsin (0.025% final concentration) was added to the PBS/EDTA and incubated for 5-15 minutes. Next either the cell containing supernatant (after settling of now "nude" carriers) were transferred (as in example 9) or the mixture of cells plus carriers were transferred (total 80 % of total mix).
After transfer of the cells to the 550 litre fermenter the remainder of the cells (hence, 10% of the viable cells) were allowed to repopulate the carriers still present in the fermenter after refilling the 50 l fermenter with culture medium.

About 70% of the cells proved to be viable

day	50 litre culture cells x 100.000/ml	250 litre culture cells x 100.000/ml
0	**8** (400 x 10^8 total)	**1.1** (275 x 10^8 total)
1		**0.8**
2		**2.9**
3		**3.4**
4		**8.9**
5		**18.0**

EXAMPLE 6

Analogous to Example 5, however, 80% of the culture of the carrier-bound cells were transferred from the mother bioreactor to the production bioreactor. Production was start after addition of virus.

The 20% of cells and carriers remaining in the mother bioreactor were trypsinised and detached and upon addition of new substrate into the mother bioreactor were allowed to repopulate the mother bioreactor while production is ongoing in the physically separated production bioreactor.

EXAMPLE 7

Large scale culture started from bulk frozen cells.

Bulk frozen cells were thawed and inoculated on a 20 litre fermenter (Cytodex carrier density 5 g/l; culture medium EfiSerf) at an inoculation density of $1x10^6$ cells/ml. After attachment, the culture medium was replaced in order to remove residual cryoprotectants.

After day 1 the amount of viable cells attached to the carriers was $0.45x10^6$ cells/ml which from then on started growth. At a density of $2.8x10^6$ cells/ml the cells were detached from their carriers by trypsinization and 80 % was transferred.

As can be deduced from the subsequent table, at day 1 the amount of viable cells after bulk freezing of cells was about 45 %.

day	cell density (x 10^6/l) in:	
	10 litre fermenter	50 litre fermenter
0	**1.0**	
1/2	**0.45**	
3/4	**1.3**	
5	**2.6**	
6	**2.8** (280 x 10^8 total)	
6	**0.6** (60 x 10^8 total)	**0.28** (140 x 10^8 total)
7		**0.4** (200 x 10^8 total)

Claims

1. Method for the preparation of cells for use in the production of biologicals, by culturing cells from a working seed stock by one or more passages up to a defined maximum number of passages into a preproduction batch in a mother bioreactor where after from the mother bioreactor in repeating steps:
(1) part of the content of the mother bioreactor is transferred to one or more production bioreactors, and
(2) the remaining content of the mother bioreactor is used as a seed for subsequent preparing of a preproduction batch.

2. Method according to claim 1, characterised in that in the one or more production bioreactors the biological of interest is produced.

3. Method according to claim 1 or 2, characterised in that the cells are anchorage-dependent, the cells are grown on a solid support, and prior to each transfer step from one bioreactor to an other bioreactor the cells are released from the solid support.

4. Method according to claim 1-3, characterised in that the biological of interest is a virus.

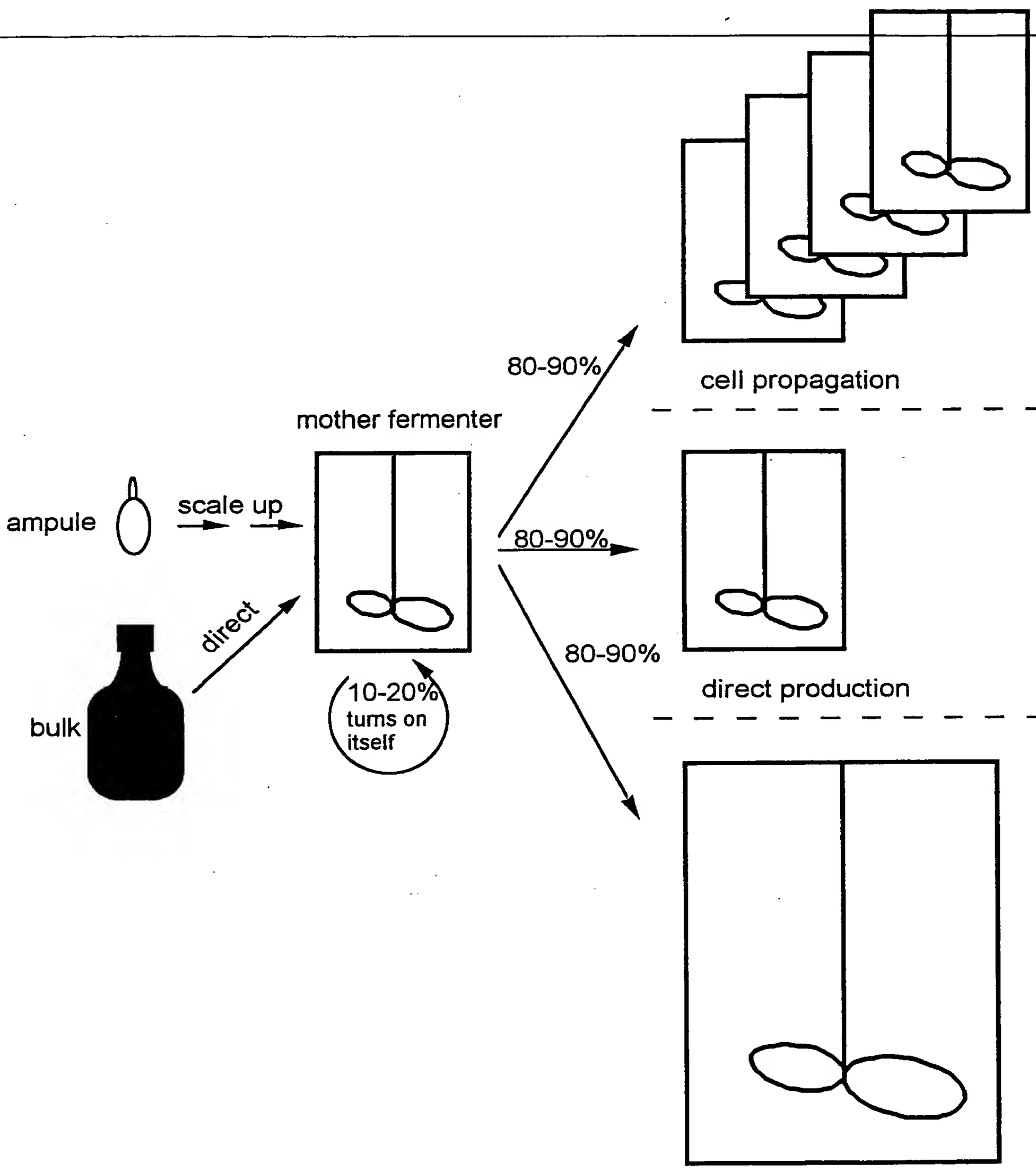
Abstract

The present invention is concerned with a method for the preparation of cells (in particular anchorage-dependent cells) for use in the production of biologicals (such as viruses), by culturing cells from a working seed stock by one or more passages up to a defined maximum number of passages into a preproduction batch in a mother bioreactor where after from the mother bioreactor in repeating steps:

(1) part of the content of the mother bioreactor is transferred to one or more production bioreactors, and

(2) the remaining content of the mother bioreactor is used as a seed for subsequent preparing of a preproduction batch.

FIGURE 1


Daughter fermenters
actions and carriers
80-90%
cell propagation
mother fermenter
ampule
scale up
80-90%
direct
80-90%
direct production
bulk
10-20%
turns on
itself
cell propagation
add new carriers